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Ataxia-telangiectasia (AT) is an autosomal recessive disorder characterized by neurologic and other abnormalities as well as an approximately 100-fold increase in cancer incidence. A single gene, *ATM*, appears to be mutated in the majority of AT families. *ATM* functions as a central component of the cellular response to DNA damage. It has been postulated that heterozygotes for *ATM* mutations have an increased susceptibility to radiation-induced breast cancer. To test this hypothesis we performed a study to test the frequency of *ATM* mutations in woman with breast cancer after therapeutic radiation therapy for Hodgkin's disease. Cases were compared to matched controls treated for Hodgkin's disease with no breast cancer. We observed no truncating mutations of *ATM* in either group, and no difference in the rate of missense mutations (37% controls vs 21% cases). Some missense mutations were observed at polymorphic (>1%) frequencies. Further studies analyzing the frequencies of these mutations in the general population, as well as functional studies, will be necessary to resolve their potential role as susceptibility alleles for lymphoid cancers.

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Kenneth Gift 30 July 00

PI - Signature Date

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I. INTRODUCTION

Ataxia-telangiectasia (AT) is an autosomal recessive disorder characterized by neurologic abnormalities (cerebellar degeneration, ataxia, and progressive mental retardation), dilation of blood vessels (oculocutaneous telangiectasia), immune deficiencies, and premature aging.[1] AT homozygotes also suffer from an approximately 100-fold increase in cancer incidence, with leukemias and lymphomas being particularly common.[2-4] A single gene, *ATM*, appears to be mutated in the majority of AT families studied to date.[5] The exact function of the *ATM* gene product is unknown, but the coding sequence bears similarity to yeast genes that serve cell cycle checkpoint and DNA repair functions.[6-9] Evidence suggests that *ATM* functions as a central component of the cellular response to DNA damage.[10]

AT heterozygotes do not manifest the multisystem abnormalities characteristic of the homozygous state. However, these individuals may also be prone to develop malignancies. In a large prospective follow-up study of 161 AT families, the risk of all cancers in female heterozygotes was 3.5 times that of non-carriers.[11] In particular, heterozygous females appeared to have at least a 5.1 times excess risk of breast cancer. Smaller, retrospective studies have also indicated that these individuals may suffer from an increased risk of cancer in general and female breast cancer in particular.[4,12,13] The issue of AT heterozygote cancer susceptibility is not trivial. Although AT is a rare disease, the population frequency of AT heterozygosity is estimated to be 1.4%, and Swift has speculated that up to 8% of all breast cancers in the United States may occur in women who are carriers of an abnormal AT allele.[12,14] The true prevalence of *ATM* mutation among unselected women with breast cancer is probably considerably lower than this figure. Using an exon-scanning PCR single-strand conformation polymorphism (PCR-SSCP) assay, Vorechovsky *et al* were unable to detect any *ATM* mutations in 38 unselected primary breast cancer cases.[15] A recent analysis of 401 women with early-onset breast cancer demonstrated a mutation frequency of only 0.5%.[35] Even accepting the limitations of this method and the possible existence of intronic or regulatory mutations, it is unlikely *ATM* mutations are present in a significant fraction of unselected women with breast cancer. However, because of design limitations, the available studies are unable to exclude a significant relative risk associated with the inheritance of an *ATM* mutation, particularly if a second insult is required to unveil the inherited susceptibility.

It remains possible that women who carry a mutant *ATM* allele are predisposed to breast cancer after exposure to a particular environmental factor, specifically radiation. Swift and his coworkers have suggested that diagnostic, therapeutic, or occupational exposure to radiation may predispose heterozygotes to the development of cancer.[11] This observation is biologically consistent with reports that cultured fibroblasts from AT heterozygotes suffer from an *in vitro* defect in the cellular response to radiation damage.[16,17] Unfortunately, the available studies suffer from significant methodological limitations and the conclusions are by no means universally accepted.[18] A creative approach is required to prove or refute Swift's hypothesis.

If women carrying a single mutant *ATM* allele are indeed susceptible to the genotoxic effects of radiation, one would expect to see an excess number of AT heterozygotes among women with radiation-induced breast cancer. The identification of such women is generally problematic due to difficulties in the quantitation of radiation exposure and in the establishment of a cause-effect relationship between that exposure and the subsequent development of malignancy. However, one well-defined group that does appear to be prone to develop radiation-associated breast cancer is women receiving therapeutic irradiation for Hodgkin's disease (HD). Several large studies have determined that women receiving radiotherapy for HD have a relative breast cancer risk of 1.3-2.2 when compared to controls.[19-26] The latency period is quite long, and the risk appears to be most significant after 15 years of follow-up.[20,21,24] Women who receive their radiotherapy before the age of 30 appear to be more prone to develop radiation-associated breast cancer, and those treated during adolescence have the greatest risk of all.[20,21,27] In a series collected at Stanford University, woman receiving radiotherapy between the ages of 10 and 19 years were 39 times more likely than controls to develop breast cancer in their third decade.[21] Interestingly, this relative risk correlates with an absolute risk of approximately 1.6%,[28] which is similar to the projected frequency of heterozygosity for *ATM* mutation in the general population.

Yahalom *et al* had previously identified 37 patients treated at MSKCC for breast cancer occurring after radiotherapy for HD.[29] We propose to study this cohort for the presence of *ATM* mutations and to perform a case-control to define the risk of breast cancer associated with such mutations. This novel approach tested the hypothesis that *ATM* mutations predispose women to breast cancer after radiation exposure. The approach was particularly innovative because it utilized a cohort with defined radiation-associated cancer, and was not hindered by the difficulties inherent in studies that attempt to retrospectively attribute cancer to radiation exposure.

This report constitutes the final report, including a one year no-cost extension of this award. The report summarizes additional findings based on additional experiments not included in the original study design or scope of work, but which grew out of preliminary findings of the 1999 Report.

II. BODY

A. Protocol Submission and IRB Approval

After DAMD17-97-1-7147 was awarded, the full protocol was submitted to the Memorial Sloan-Kettering Institutional Review Board for approval. Formal approval for the study was obtained on June 24, 1997 and the study was assigned local protocol number 97-81. Patient ascertainment and recruitment began immediately and was continued during the year of the no-cost extension.

B. Subject Ascertainment/Recruitment

We now report on thirty seven patients with histories of both breast cancer and Hodgkin's disease that were identified from an original cohort of women seen at MSKCC or from collaborating centers. The University of Pennsylvania has contributed a single case to the study at this point in time. A control group of 26 patients were identified from the files of patients undergoing radiation therapy for Hodgkin's disease at MSKCC. Controls were matched to cases with respect to age, stage, radiation ports and dose received, and time of diagnosis of Hodgkin's disease. Cases differed from controls in that they developed breast cancer after diagnosis of Hodgkin's disease.

All patients had undergone genetic counseling, provided informed consent, had donated a blood sample. In addition, the parents of a child with ataxia-telangiectasia have donated samples to serve as positive controls for the mutation detection techniques being developed.

C. Laboratory Methods

1. Creation of Lymphoblastoid Cell Lines

To facilitate mutation detection, lymphoblastoid cell lines have been established from peripheral blood lymphocytes for 30 cases by Epstein-Barr-virus transformation.[36]

2. Protein Truncation Testing (PTT) Analysis

PTT presents several advantages when compared to other mutation screening methods. Large gene segments of 2-3Kb can be analyzed at the same time in one reaction and mutations that lead to truncated protein products are efficiently detected.[37,38] Since more than 70% of the mutations in the ATM gene lead to truncated proteins, we chose PTT for initial screening of patient-derived cDNAs.

cDNA Preparation.

Total RNA was then isolated from the lymphoblastoid cell lines using the Ultraspec RNA isolation system (Biotecx Laboratories, INC., Houston, TX) according to manufacturer's instructions. RNA pellets were resuspended in 100μl of DEPC treated water. The total RNA was reverse transcribed with random hexamers using the Superscript cDNA Preamplification Kit (Life Technologies) to generate cDNA. Reverse transcriptions were carried out in a volume of 20μl containing 1-3 μg of RNA, 100 ng Random Hexamers, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 0.5 mM dNTP, 10 mM DTT, and 200U Superscript II Reverse

Transcriptase. The reaction tubes were incubated at 42°C for 60 min and then 70°C for 15 min to terminate the reaction. The product was treated with 2U E.coli Rnase H and incubated at 37°C for 20 min. The cDNA was then diluted with 20µl sterile water and stored at -20°C. A 2.5µl aliquot of cDNA was used as a subsequent PCR template. The entire coding region of the ATM transcript, composed of 63 exons covering 9.2kb, was divided into 7 overlapping regions. RT-PCR was used to produce transcription templates for PTT. Total RNA was isolated from lymphoblastoid cell lines using the Ultraspec RNA isolation system (Biotecx Laboratories, INC., Houston, TX) according to manufacturer's instructions. RNA pellets were resuspended in 100µl of DEPC treated water. The total RNA was reverse transcribed with random hexamers using the Superscript cDNA Preamplification Kit (Life Technologies) to generate cDNA. Reverse transcriptions were carried out in a volume of 20ul containing 1-3 µg of RNA, 100 ng Random Hexamers, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 0.5 mM dNTP, 10 mM DTT, and 200U Superscript II Reverse Transcriptase. The reaction tubes were incubated at 42°C for 60 min and then 70°C for 15 min to terminate the reaction. The product was treated with 2u E.coli Rnase H and incubated at 37°C for 20 min. The cDNA was then diluted with 20µl sterile water and stored at -20°C. A 2.5ul aliquot of cDNA was used as a subsequent PCR template.

Primers.

The entire coding region of the 9.2 Kb of ATM transcript and the adjacent untranslated region was divided into 7 overlapping regions (e, f, g, a, b, c, and d) and each segment was analyzed separately. Forward primers were designed to include a T7 promoter sequence for the initiation of transcription by T7 RNA polymerase and an ATG sequence for initiation of translation Primers being used for the PTT analysis are shown in Table 1. along with the optimal annealing temperatures.

RT-PCR.

PCR of each region was carried out in a volume of 50µl containing 1.5 mM $MgCl_2$, 20 mM Tris-HCl (pH 8.0), 50 mM KCl, 200 µM dNTP mix, forward and reverse primers at 10 pM, and 3.75U of Platinum Taq DNA Polymerase (Life Technologies). PCR of each region included of an initial denaturation of 3 min at 94°C, 35-40 cycles of 1 min at 94°C, 1 min at 50-56°C, 3 min at 72°C and a final extension step for 10 min at 72°C.

PTT Analysis

A 25µl reaction mix containing 2-5µl of RT-PCR product, 12.5µl of TnT rabbit reticulocyte lysate (Promega),3.5µl of master mix containing 1.0µl of [^{35}S] methionine (1000 Ci/mmol, NEN Life Science), 0. 5µl of RNAsin (40u/µl), 0.5µl of amino acid mix, 0.5µl of RNA polymerase and 1µl of TnT buffer was be incubated at 30°C for 1.5 hr. Following the PTT, 5µl of product was electrophoresed on 12.5 % SDS-polyacrylamide gels. Following the electrophoresis, the gel was soaked in Amplify solution (Amersham Life Science) for half an hour, dried and subjected to autoradiography. Figure (1) shows a representative picture of the expected size protein fragments in regions B and D in four of the subjects.

Methodology for detection of the missense mutation 5557 G/A transition in exon 39 in the control population.

In the course of this study, a particular missense mutation (5557G/A) was detected at increased frequency in both cases and controls (*vide infra*). To determine the frequency of this mutation in an unaffected control population a 184 bp segment of the exon 39 was amplified using the following primers:

Forward primer 5' TTTAATATGTGTCAACGGGGCA 3'

Reverse Primer 5' CAGATTTCTCCATGATTCATTTAGA 3'

The reverse primer was designed such that it created a unique Bgl II restriction site (A'GATC) in the normal sequence, whereas this site was abolished in the mutant sequence in which the G/A transition occurred. A 25 µl PCR reaction containing 50 ng of DNA in PCR buffer containing 60mM Tris–HCl, 15 mM Ammonium sulphate,2 mM $MgCl_2$ 0.25 mM dntps, 0.2µ M of the primers and 1U of Taq. polymerase. Amplifications were performed using a DNA thermal cycler 9600(Perkin Elmer Cetus) for approximately 40 cycles at 55°C annealing temperature, followed by running 10 µl of the product on a 3% agarose gel to check for amplification. A second 10 µlaliqout of the PCR product was digested with 10U of BglII (New England Biolabs) at 37°C for 3-4h. The digestion products were run on 3% nusieve GTG agarose (FMC products) . The restriction digestion yielded a 157 and 27 bp products for a normal genotype and a 184 bp product in case of the mutant. (Fig.3). This analysis resulted 26 mutants (20.5%) out of the 127 controls screened.

Mutant DNA samples were amplified by PCR as described above and purified using a Qiaquick PCR purification kit by Qiagen. Purified PCR product was used as a template for sequencing using the

dideoxynucleotide chain termination method using the fmol DNA sequencing system by Promega. The reaction products were analyzed on a 8% polyacrylamide /7M urea/.1M Trisborate/2.5mm EDTA gels. After electrophoresis, the gel was transfererd to blotting paper, dried and subjected to autoradiography. Fig (2). Shows some representative mutants with a G/A transition, along with the negative and the positive controls.

Sequencing Approach

Sequence analysis was conducted. DNA was extracted by the ProteinaseK/SDS method. cDNA was prepared from RNA by reverse-transcription reaction with Superscript reverse-transcriptase (Gibco-BRL). cDNA was amplified with Expand Long Template PCR system (Boheringer Mannheim) with primer *ATM*F and *ATM*R. The amplified cDNA was divided into two sub fragments (*ATM* RA and *ATM* RB) by the following primers (*ATM*in,AR and *ATM*BF, *ATM*out, respectively). The two cDNAs were then directly sequenced. Sequencing was preformed on a ABI377 sequencer with BigDyes modified dNTPs.Sequence chromatograms were analyzed with Sequencher software (Gene codes corporation). Whenever a mutation was found in the cDNA, the corresponding genomic DNA region was amplified with the appropriate primers and sequenced for confirmation. Sequence analysis was done in collaboration with colleagues in the laboratory of Dr. Y. Shiloh, who originally identified the ATM gene, and in concert with efforts at QBI laboratories.

Results

D. Results

Aim 1. Identification of cases of women who had developed breast cancer after exposure to therapeutic chest irradiation for Hodgkin's disease, and identify appropriate controls.

As projected in the initial design, we have been successful in recruiting 37 patients with breast cancer following therapeutic radiation. An additional case were ascertained from the University of Pennsylvania. Twenty six matched controls were identified from MSKCC, and one from the University of Pennsylvania. Controls were matched for date of birth (within 3 years), date of diagnosis of Hodgkin's disease (within 5 years, age at time of radiation therapy (within 4 years), radiation dose (within 40 Gy).

Aim 2: Identify women in the cases and controls who are heterozygous for germline ATM mutations

Results of PTT analysis

No protein truncating mutations were identified in 37 cases or 26 controls

Results of Full Sequence Analysis

Fourteen missense mutations were detected in 14 of the cases and 24 of the controls. Seven mutations were detected in more than one patient. Seven of these mutations have nor previously been reported. Seven mutations are represented in prior publications or on-line databases (www.vmresearch.org/*ATM*ut-t.htm).

New mutations not previously reported include: G514D, P604S, F763L, F1463C, T1696A, C1821W, and L2307F. Of these P604S creates a new glycosylation site, C1821W does not alter a known glycosylation site, whilst the other five mutations occur in unknown domains. Of the seven mutations previously described, four (S707P, F858L, P1053R, and L1420F are listed in on-line databases as known to be or likely to be deleterious to *ATM* function. S707P was observed in an early-onset breast cancer case described by Izatt et al., 1999; however there was no family history of breast cancer in this kindred. This mutation occurs in a conserved region in the murine *ATM* which shows 91% amino acid similarity to human *ATM* protein. In addition we predict that the S707P abolishes a putative glycosyation site. F858L was also previously observed in 1 of 200 early-onset breast cancers and 1/100 normal controls (Izatt et al., 1999) and was characterized as a rare sequence variant by Sandoval et al, 1999 where it was observed at an allele frequency of 0.02. (These are references 40-42 in the Refereces section). P1053R was observed in 1 of 38 breast cancer patients and 5 of 224 (2.3%) chromosomes of European Caucasians (Verechovsky, 1996) as well as AT families, where it was classified as a rare variant seen at allele frequency of 0.07. F858L was also seen in patient with B cell CLL where it was associated with partial inactivation (65% expression of *ATM* protein) (Stankovic et al., 1999)[42]. L1420F was observed in 1 of 38 in breast tumors and 4 of 224 (1.8%) control chromosomes (Vorechovsky et al., 1996) in one series and 2/200 cases and 0/100 controls in another series (Izatt et al., 1999). Three mutations previously reported are classified as neutral polymorphisms in the *ATM* database. D1853N was previously reported by Sandoval et al 1999 at an allele frequency of 0.18, and by Hacia et al, 1998. We also confirmed that this mutation was a common polymorphism (see below). D1853V was also reported by Sandoval et al at allele frequency of .03. Finally, V2079I was observed in 1 of 38 breast tumors and 3 (1.3%) of 224 chromosomes from European Caucasians (Vorechovsky et al, 1996).

With the exception of the D1853N, all of the mutations seen in the Hodgkin's disease patients were present in 1% or less of the healthy control group (Table 1). Excluding the D1853N variant, of the 37 cases with Hodgkin's disease followed by breast cancer, 7 individuals carried an *ATM* missense mutation; three cases carried two mutations. One variant, the was observed in homozygous state.Of the 26 Hodgkin's cases without breast cancer, 10 of the 26 patients carried an *ATM* missense mutation; two patients carried two mutations, one carried three mutations and one carried four mutations.

Aim 3: Determine relative risk of radiation associated breast cancer associated with the presence of a germline ATM mutation

The results of the PTT analysis and the preliminary results of the sequence analysis show no significant difference of the prevalence of mutations in cases or controls.

Based on preliminary power calculations, assuming a heterozygote frequency of 1.4% in the general population, and based on a sample size of 30 cases and controls, and the absence of detection of truncating mutations of ATM in the Hodgkin's breast cancer group rules out a large effect of the ATM mutations (relative risk of 22).

Since missense mutations were seen at approximately equal proportion is cases and controls, it is concluded that these variants also do not confer a radiation sennsitivity to Hodgkin's disease.

E. Unexpected findings, and Additional studies in progress.

The finding of the relatively high frequency of missense mutations in both cases and controls was unanticipated. Some of the missense mutations observed have been observed previously. Thus, the mutation 2572 T/C, 3161 C/G, and 6235 G/A had previously been observed in breast tumors, and in B cell cancers.[17]

The findings of this project argue against a substantial predisposition to radiation-induced breast cancer resulting from ATM mutations (fulfilling the primary specific aim of this proposal). However, the finding of substantial numbers of missense mutations among women with Hodgkin's Disease (with and without breast cancer) raises the possibility that such mutations may confer a susceptibility to Hodgkin's disease. In order to investigate this hypothesis, a control series of 127 unmatched samples from normal donors was obtained in collaboration with the New York Blood Center. These samples were available as part of an unrelated study as part of an ongoing protocol that allowed genotyping of anonymized DNA samples. Dr P. Kolachna utilizing reagents supported by this project, devised a method of mutation detection for the most common polymorphism 5557G/A (see Methods section). This analysis confirmed the presence of this mutation in 26/127 (20.5%) of cases studied. These data confirm that this mutation is a common polymorphism of ATM, and unlikely to be related to lymphoma risk.

Determination of the frequency of the other missense changes in a panel of controls matched for ethnic status are outside the scope of work of the initial proposal but have been nearly completed as of this date.

These results revealed that the 1541 G/A (G514D) variant was seen in 1/96 normal chromosomes, the 2289 T/A (F763L) in 0/46 chromosomes, the 2572 T/C(F858L) in 0/144 chromosomes, the 3161 C/G (1053R)1 in 1/182 chromosomes, the 4258 C/T (L1420F) iin 0/160 chromosomes, the 5557 G/A (D1853N) in 16/196 chromosomes, the

5558 A/T (D1853V) in 2/196 chromosomes, the 6235 G/A (V2079I) in 0/104 chromosomes and the 6919 C/T (L2307F) in 1/130 chromosomes. Interestingly in no case in the control population were compounds observed.

III. KEY RESEARCH ACCOMPLISHMENTS

This study has:

- Assembled a cohort of women and DNA repository of subjects with breast cancers occurring after radiation therapy for Hodgkin's disease

- Shown that there is no increase in truncating mutations of ATM in this cohort of women, suggesting that the increase in breast cancer risk attributable to a germline ATM mutation, if extant, is not very large.

- Demonstrated that there is a significant proportion of missense mutations of in both the cases and controls (i.e. patients with Hodgkin's Disease). This finding suggests that ATM mutations are not associated with increased risk due to exposure to ionizing radiation. However, the presence of compound heterozygotes for these variants may be associated with an increased risk for Hodgkin's disease, breast cancer, and possibly other cancers. Additional experiments—both epidemiological (case control) and molecular genetic (functional studies of mutations) will be necessary to resolve these issues.

IV. REPORTABLE OUTCOMES

This results of this study have been prepared as a manuscript for submission to the journal BLOOD as a rapid communication. Submission will be made at the time this report is being study is returned, and DAMD support will be recognized.

V. CONCLUSIONS

Within the power constraints defined in the initial proposal, a search for truncating mutations of the ATM gene, as well as a separate full-sequencing analysis performed in collaboration with the investigator who cloned the ATM gene, did not reveal any difference in incidence of ATM mutations in women exposed to ionizing radiation who developed breast cancer after Hodgkin's disease, and matched controls with the same exposure who did not develop Hodgkin's disease. These data are consistent with those recently published by another group.[43] These data argue against a large radiation-associated risk conferred by inheritance of a germline ATM mutation. This project did reveal several missense changes of the ATM gene, some at polymorphic frequencies in Hodgkin's disease cases with and without breast cancer. In addition, a number of

cases were compound heterozygotes for several of these mutations, observed individually at frequencies of 1-2% in controls. These findings suggest that these ATM mutations may represent hypomorphic alleles, and are similar to findings we have reported over the past year for rare alleles of the type I TGFbeta receptor.[44] The potential significance of ATM mutations as predisposing alleles for human cancers will require additional epidemiologic and functional molecular analysis.

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Table 1. PTT[a] Primers being used for Mutation Screening of the ATM Gene

NAME OF THE PRIMER	NUCLEOTIDE SEQUENCE	TM[0] C[b]	FRAGMENT SIZE (bp)	REGION AMPLIFIED
ATMe	Forward(T7)[c]-GAAGTTGAGAAATTTAAGC Reverse AATGCAACTTCCGTAAGGC	50.0°	1316	76-1392
ATMf	Forward(T7)-GCAGATATCTGT Reverse GTAGGTTCTAGCGTGCTAGA	55.0°	1769	1048-2817
ATMg	Forward(T7)-AATGACATTGCAGATATTT Reverse TCAGTGCTCTGACTGGCACT	55.0°	1655	2437-4092
ATMa	Forward(T7)-ACGTTACATGAGCCAG Reverse TCCAAATGTCATGATTTTCAC	50.0°	1387	4048-5435
ATMb	Forward (T7)-CTGGCCTATCTACAGC Reverse CAACCTGCTAAGTGTGGGAT	55.0°	1247	5282-6529
ATMc	Forward (T7)-CAGTGGGACCATTGC Reverse TTCTGACCATCTGAGGTCTCC	55.0°	1534	6322-7856
ATMd	Forward (T7)-GATCACCCCATCACA Reverse TCACACCCAAGCTTTCCATC	55.0°	1521	7651-9172

a Protein Truncating Test

b Annealing Temperature

c Promoter sequence and Translation start codon were added to all the forward primers at their 5' end GGATCCTAATACGACTCACTATAGGAACAGACCATG.

Figure 1 : Mutation screening by Protein Truncation in regions B and D. Autoradiogram of ^{35}S-labeled protein fragment show the resolution of a 57.77 and 46.29 kD normal protein fragment in 4 of the subjects tested in the first 8 lanes., followed by a negative control and luciferase as a positive control.

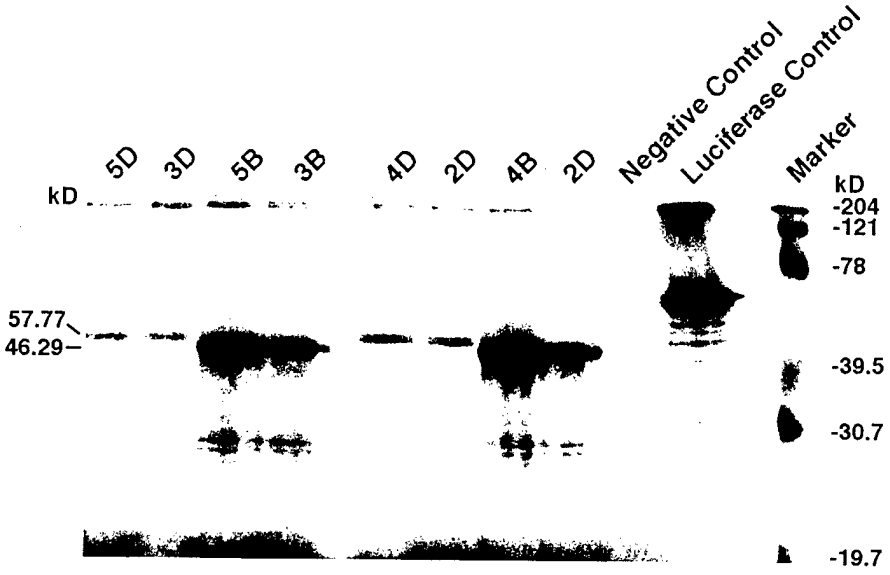

Figure 2 PCR amplified exon 39 product of normal controls restriction digested with Bgl II and run on 3% gel. Figure shows. the negative control with a 157 bp band and the positive control with the 184 bp band in the right hand panel. The 27 bp band is not visible. The left hand panel shows the digestion products of some of the control population samples. #172, 177 and 181 are positive for the mutation, where as the remaining ones are negative.

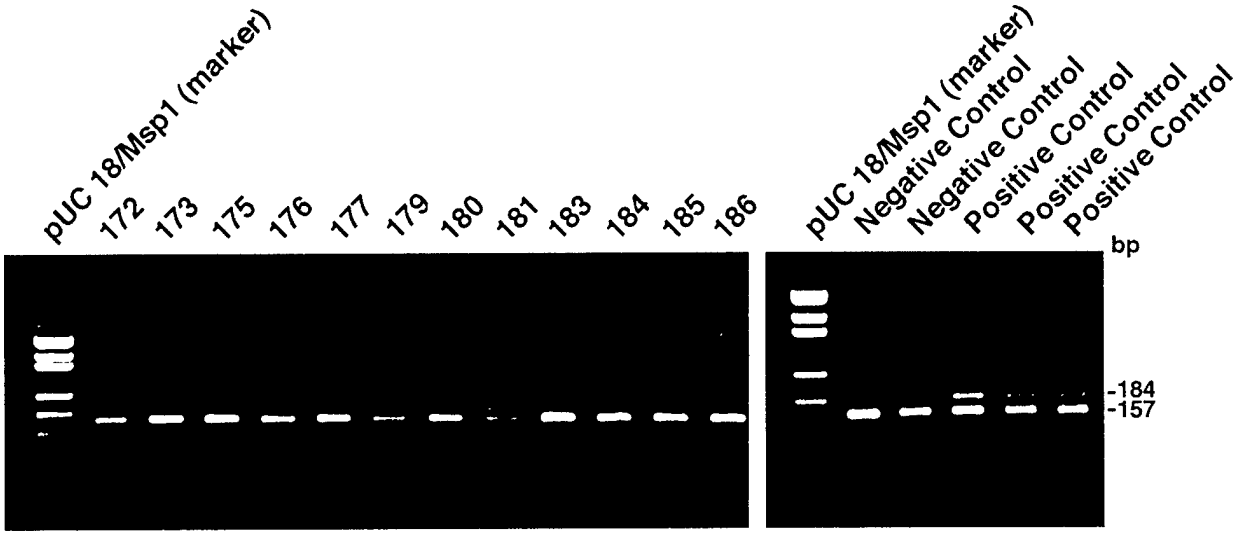

Figure 3 shows the G/A transition at position 458 leading to stretch of 4 A's in case of the positive controls and some of the mutants identified.(arrow), and the absence of A in the negative control.